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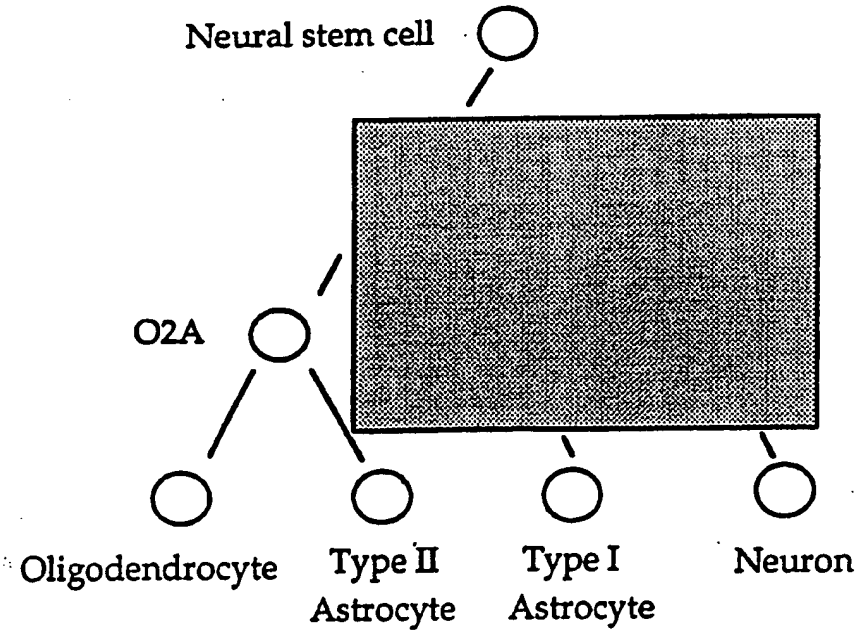

(57) Abstract

A method for the remyelination of neurons is disclosed wherein neural stem cells isolated from adult or fetal neural tissue are proliferated in a culture medium containing a growth factor to produce precursor cells having a nestin (+) phenotype. The precursor cells are capable of differentiation into oligodendrocytes which, when associated with a demyelinated neuron, effect remyelination.

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REMYELINATION USING NEURAL STEM CELLS

FIELD OF THE INVENTION

The present invention is directed to the uses of neural stem cells (NSCs) and their descendants to remyelinate axons. More particularly, the invention is related to the treatment of demyelinating diseases by the remyelination of neurons through the addition of exogenous myelin forming cells and precursors thereof.

BACKGROUND OF THE INVENTION

Myelin is a cellular sheath, formed by glial cells, that surrounds axons and axonal processes that enhances various electrochemical properties and provides trophic support to the neuron. Myelin is formed by Schwann cells in the peripheral nervous system and by oligodendrocytes in the central nervous system.

Demyelination of central and peripheral neurons occurs in a number of pathologies and leads to improper signal conduction within the nervous systems. Among the various demyelinating diseases Multiple Sclerosis (MS) is the most notable.

In both human demyelinating diseases and rodent models there is substantial evidence that demyelinated neurons are capable of remyelination in situ. In MS, for example, it appears that there are often cycles of de- and remyelination. Similar observations in rodent demyelinating paradigms lead to the prediction that exogenously applied cells would be capable of remyelinating demyelinated axons. This approach has proven successful in a number of experimental conditions [Freidman et al., Brain Research, 378:142-146 (1986); Raine, et al., Laboratory Investigation 59:467-476 (1988); Duncan et al., J. of Neurocytology, 17:351-360 (1988)]. The sources of cells for some of these experiments included dissociated glial cell suspensions prepared from spinal cords (Duncan et al., supra), Schwann cell cultures prepared from sciatic nerve [Bunge et al., 1992, WO 92/03536; Blakemore and Crang, J. Neurol. Sci., 70:207-223 (1985)]; cultures from dissociated brain tissue [Blakemore and Crang, Dev. Neurosci. 10:1-11 (1988)], oligodendrocyte precursor cells [Gumpel et al., Dev. Neurosci. 11:132-139 (1989)], O-2A cells [Wolswijk et al., Development 109:691-608 (1990); Raff et al., Nature 3030:390-396 (1983); Hardy et al., Development 111:1061-1080 (1991)], and immortalized O-2A cell lines, [Almazan and McKay Brain Res. 579:234-245 (1992)].

O-2A cells are glial progenitor cells which give rise in vitro only to oligodendrocytes and type II astrocytes. Cells which appear by immunostaining in vivo to have the O-2A phenotype have been shown to successfully remyelinate demyelinated neurons in vivo. Godfraind et al., J. Cell Biol. 109:2405-2416 (1989). Injection of a large number of O-2A cells is required

to adequately remyelinate all targeted neurons in vivo, since it appears that O-2A cells (like other glial cell preparations) do not continue to divide in situ. Although O-2A progenitor cells can be grown in culture, currently the only available isolation technique employs optic nerve as starting material. This is a low yield source, which requires a number of purification steps. There is an additional drawback that O-2A cells isolated by the available procedures are capable of only a limited number of divisions. Raff Science 243:1450-1455 (1989).

For in vivo remyelination, it would be advantageous to inject only a small number of cells which could go on to divide, migrate to appropriate targets, and differentiate in situ. It has been shown that the presence of type I astrocytes is important for remyelination to occur with exogenously added oligodendrocytes. The use of oligodendrocyte precursors such as O-2A cells requires that type I astrocytes be co-injected, or alternatively that platelet-derived growth factor (PDGF) be provided to the remyelinating cells. PDGF is a potent mitogen for the O-2A precursor and is secreted from type 1 astrocytes.

Crude cell preparations and suspensions are not preferred as exogenous sources of remyelinating cells because they contain an unpredictable number of cells as well as large numbers of both neural and non-neural cell types, thus making reproducibility of the process difficult. It is also difficult to obtain suitable numbers of cells from these preparations.

Transformed O-2A cell lines are unsuitable for transplantation due to the fact that the transformation

process leads to a genetically (oncogene) controlled cell division as opposed to primary cell lines or neural stem or progenitor cells where regulation of division is at an epigenetic level. Additional potential problems include instability of cell lines over long periods of time, and aberrant patterns of differentiation or responses to growth factors. Goldman Trends Neuro. Sci. 15:359-362 (1992).

Thus there exists a need for a reliable source of cells for remyelination therapy. Preferably cellular division in such cells from such a source would be epigenetically regulated and a suitable number of cells could be efficiently prepared in sufficient numbers to effect remyelination. The cells should be suitable in autografts, xenografts, and allografts without a concern for tumor formation.

Accordingly, it is an object of this invention to provide a reliable source of epigenetically regulated cells for transplantation, which are capable of differentiating into oligodendrocytes.

It is another object of this invention to provide a method for treatment of myelin deficient recipients, whereby precursor cells derived from stem cells proliferated in vitro are transplanted into a myelin deficient recipient where the cells differentiate into oligodendrocytes thus effecting remyelination of the recipient's axons.

These and other objects and features of the invention will be apparent to those skilled in the art from the following detailed description and appended claims when taken in conjunction with the figures.

None of the f regoing references is believed to disclose the present invention as claimed and is not presumed to be prior art. The references are offered for the purpose of background information.

SUMMARY OF THE INVENTION

A method of remyelinating neurons is described wherein isolated neural stem cells are proliferated, in vitro, in culture medium containing a growth factor which induces the production of precursor cells. The precursor cells are harvested and, under appropriate conditions, effect the remyelination of demyelinated axons. Alternatively, the precursor cells are allowed to differentiate into oligodendrocytes in the presence of a culture medium which is substantially free of the stem cell-proliferating growth factor. The precursor cell-derived oligodendrocytes are then associated with demyelinated axons to effect remyelination.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "stem cell" refers to an undifferentiated cell which is capable of proliferation and giving rise to more stem cells having the ability to generate a large number of progenitor cells that can in turn give rise to differentiated, or differentiable daughter cells.

The term "neural stem cell" (NSC) refers to the stem cells of the instant invention, the progeny of which under appropriate culturing conditions, include both glial and neuronal progenitor cells.

The term "progenitor cells" refers to the undifferentiated cells of the instant invention, derived

from neural stem cells, the progeny of which may, under appropriate conditions, include glial and/or neuronal progenitor cells.

The term "oligodendrocyte" refers to a differentiated glial cell which forms the myelin surrounding axons in the central nervous system (CNS). Oligodendrocytes are of the phenotype galactocerebroside (+), myelin basic protein (+), and glial fibrillary acidic protein (-) [Gal C(+), MBP(+), GFAP(-)].

The term "type I astrocyte" refers to a differentiated glial cell type with a flat protoplasmic/fibroblast-like morphology that is GFAP(+), Gal C(-), and MBP(-).

The term "type II astrocyte" refers to a differentiated glial cell displaying a stellate process bearing morphology of the phenotype GFAP(+), Gal C(-), and MBP(-).

The term "neuronal progenitor cells" refers to cells which produce daughter cells which under the appropriate conditions become or give rise to neurons.

The term "oligodendrocyte precursor cells" refers to cells which give rise to oligodendrocytes. Oligodendrocyte precursor cells can have the phenotype A2B5(+), O4(+)/Gal C(-), MBP(-) and GFAP (-) [but are not limited to this phenotype].

The term "neurosphere" refers to a cluster of cells derived from neural stem cells and cultured <u>in vitro</u>. At least some of the cells are of the nestin (+) phenotype. The cluster is comprised of stem cells

and/or progenitor cells and may or may not include differentiated cells.

The term "precursor cells" refers to the living cells of the instant invention that are derived from neural stem cells proliferated in a culture medium containing a growth factor, and includes both progenitor and stem cells. Precursor cells typically grow in the form of neurospheres, but may exhibit different growth patterns depending upon culture conditions.

The term "growth factor" refers to a protein or peptide having a growth or trophic effect.

The term "donor" refers to the human or animal which is the source of the neural stem cells used in the instant invention.

The term "harvesting" refers to any method used to procure proliferated cells in a form suitable for injection or transplantation.

The term "recipient" refers to the human or animal that has demyelinated axons and into which the precursor cells or oligodendrocytes derived from the precursor cells are transplanted or injected.

Brief Description of the Drawings

Figure 1 indicates only a single progenitor cell for oligodendrocytes and type II astrocytes. In fact, various reports indicate there may be multiple progenitor cells for these differentiated phenotypes. Goldman, TINS 15:359-362 (1992). An important aspect of the diagram is that n ural stem cells are unique in

that they are the only stem cell capable of giving rise to both neurons and glial in vitro.

Description of the Preferred Embodiments

Phenotypical characteristics

Neural stem cells (NSCs) have been reported and their potential use described. (Reynolds and Weiss, Science 255:1707 (1992)). NSCs have been shown to give rise to at least three glial phenotypes including oligodendrocytes and type I and II astrocytes. NSCs also give rise to neuroblasts. (Reynolds and Weiss, Restorative Neurology & Neuroscience 4:208 (1992)).

Neural stem cells can be isolated and cultured by the method of Reynolds and Weiss (supra). In brief, the epidermal growth factor (EGF) responsive stem cell, when grown in a defined serum-free medium, and in the presence of a mitogen such as EGF or the like, is induced to divide giving rise to a cluster of undifferentiated cells. The cluster of cells are not immunoreactive for GFAP, neural filament (NF), neuron specific enolase (NSE) or MBP. However, precursor cells within the cluster are immunoreactive for nestin, an intermediate filament protein found in undifferentiated CNS cells. The nestin marker was characterized by Lehndahl et al., Cell 60:585-595 (1990), and is incorporated herein by reference. None of the mature phenotypes associated with the four cell types which may be differentiated from the progeny of the precursor cells have the nestin phenotype.

In the continued presence of a mitogen such as EGF or the like, precursor cells within the neurosphere continue to divide resulting in an increase in the size

of the neurosphere and the number of undifferentiated cells [nestin(+), GFAP(-), NF(-), NSE (-), MBP (-)]. At this stage the cells are non-adherent and tend to form the free-floating clusters characteristic of neurospheres. However, culture conditions may be varied so that while the precursor cells still express the nestin phenotype, they do not form the characteristic neurospheres. After removal of the mitogen the cells adhere to the substrate (poly-ornithine-treated plastic or glass), flatten, and begin to differentiate into neurons and glial cells. At this stage the culture medium may contain serum such as 0.5-1.0% fetal bovine serum (FBS). Within 2-3 days, most or all of the precursor cells begin to lose immunoreactivity for nestin and begin to express intermediate filaments specific for neurons or for astrocytes as indicated by immunoreactivity to NFL or GFAP respectively. In addition, a large number of cells that do not express either of these intermediate filament markers, begin to express markers specific for oligodendrocytes in a correct temporal fashion. That is, the cells first become immunoreactive for O4 (a cell surface antigen), galactocerebroside (Gal C, a myelin glycolipid) and finally, myelin basic protein (MBP). These cells also possess a characteristic oligodendrocyte morphology. This information considered in light of the oligodendrocyte progenitor cells identified by Raff gives rise to a number of possible cell lineage relationships. (See Figure 1).

Preparation of Neurospheres

Neurospheres can be generated from a variety of tissues including adult or fetal neural tissue from human or animal sources. Briefly, individual stem cells are prepared from the dissociation of neural tissues.

Dissociation can be obtained using any known procedure, including treatment with enzymes such as trypsin, collagenase and the like,or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose. Low Ca^{++} aCSF contains the same ingredients except for $MgCl_2$ at a concentration of 3.2 mM and $CaCl_2$ at a concentration of 0.1 mM.

The individual cells can be placed into any known culture medium capable of supporting cell growth and proliferation, including MEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. The dissociated cells form neurospheres in the presence of a mitogen such as EGF or the like.

Differentiation of Glial Cells from Neurospheres

Astrocytes and oligodendrocytes can be differentiated from the precursor cells of the neurosphere by placing the neurospheres in a medium containing 1% fetal bovine serum in the absence of a mitogen, such as EGF or the like, for approximately 3-4 days on poly-ornithine treated glass or plastic. These cells can then be maintained in culture for a suitable time period to produce large numbers of differentiated

cells. Cultures can then be purified to a single cell type using any of the methods known to the art for the purification of specific glial cell populations. Some preferred approaches in this regard are the immunological methods of Wolswijk et al., Development 109:691-698 (1990) and those of Franklin et al., J. Neurocytology 20:420-430 (1991).

Use of Differentiated Cells

Differentiated cells in the form of oligodendrocytes that are derived from precursor cells may be injected into demyelinated target areas in the recipient. Appropriate amounts of type I astrocytes may also be injected. Type I astrocytes are known to secrete PDGF which promotes both migration and cell division of oligodendrocytes. [Nobel et al., Nature 333:560-652 (1988); Richardson et al., Cell, 53:309-319 (1988)].

Use of Non-differentiated Precursor Cells

Non-differentiated precursor cells are preferred as the cells for treatment of demyelinating diseases. Neurospheres grown in the presence of EGF can be dissociated to obtain individual precursor cells which are then placed in injection medium and injected directly into the demyelinated target region. Astrocytes can promote remyelination in various paradigms. Therefore, in instances where oligodendrocyte proliferation is important, the ability of precursor cells to give rise to type I astrocytes may be useful. In other situations, PDGF may be applied topically during the transplantation as well as with repeated doses to the implant site thereafter.

The injection of precursor cells in remyelination therapy provides a source of immature type I astrocytes at the implant site. This is a significant feature because immature astrocytes (as opposed to mature astrocytes) have a number of specific characteristics that make them particularly suited for remyelination therapy. First, immature, as opposed to mature, type I astrocytes are known to migrate away from the implant site [Lindsay et. al, Neurosci. 12:513-530 (1984)] when implanted into a mature recipient and become associated with blood vessels in the recipient's CNS [Silver et al., WO 91/06631 (1991)]. This is at least partially due to the fact that immature astrocytes are intrinsically more motile than mature astrocytes. [Duffy et al., Exp Cell Res. 139:145-157 (1982), Table VII]. Type I astrocytes differentiating at or near the precursor cell implant site should have maximal motility and thereby optimize the opportunity for oligodendrocyte growth and division at sites distant from the implant. The localization of the astrocytes near blood vessels is also significant from a therapeutic standpoint since (at least in MS) most plaques have a close anatomical relationship with one or more veins.

Another characteristic of immature astrocytes that makes them particularly suited for remyelination therapy is that they undergo a lesser degree of cell death than mature type I astrocytes. (Silver et al., supra)

Implantation

Any suitable method for the implantation of precursor cells near to the demyelinated targets may be used so that the cells can become associated with the demyelinated axons. Glial cells are motile and are known to migrat to, along, and across their neuronal

targets thereby allowing the spacing of injections. Injection methods exemplified by those used by Duncan et al. J.Neurocytology, 17:351-361 (1988), incorporated herein by reference, and scaled up and modified for use in humans are preferred. Methods taught by Gage et al. US Patent No. 5,082,670, incorporated herein by reference, for the injection of cell suspensions such as fibroblasts into the CNS may also be employed for injection of precursor cells. Additional approaches and methods may be found in Neural Grafting in the Mammalian CNS, Bjorklund and Stenevi, eds., (1985), incorporated herein by reference.

Autografts

In some instances, it may be possible to prepare precursor cells from the recipient's own nervous system (e.g.in the case of tumor removal biopsies etc,). In such instances the precursor cells may be generated from dissociated tissue and grown in culture in the presence of a mitogen such as EGF or the like, or basic fibroblast growth factor (bFGF). Upon suitable expansion of cell numbers, the precursor cells may be harvested and readied for direct injection into the recipient's CNS. In the case of demyelinating diseases with a genetic basis directly affecting the ability of the myelin forming cell to myelinate axons, it will generally not be useful to remyelinate using the recipients cells as donor cells, unless the cells have been modified in some way to insure the lesion will not continue (e.g. genetically modifying the cells to cure the demyelination lesion).

Xeno and/or allografts may require the application of immunosuppressive techniques or induction of host tolerance to insure longevity of remyelination. Local

immunosuppression is disclosed by Gruber, Transplantation 54:1-11 (1992), incorporated by reference. Rossini, US Patent No. 5,026,365, discloses encapsulation methods suitable for local immunosuppression. General reviews and citations for the use of recombinant methods to reduce antigenicity of donor cells are disclosed by Gruber (supra). Exemplary approaches to the reduction of immunogenicity of transplants by surface modification are disclosed by Faustman WO 92/04033 (1992).

Xenografts

The instant invention allows the use of precursor cells prepared from donor tissue which is xenogeneic to the host. Since the CNS is a somewhat immunoprivileged site, the immune response is significantly less to xenografts, than elsewhere in the body. In general, however, in order for xenografts to be successful it is preferred that some method of reducing or eliminating the immune response to the implanted tissue be employed. Thus recipients will often be immunosuppressed, either through the use of immunosuppressive drugs such as cyclosporin, or through local immunosuppression strategies employing locally applied immunosuppressants. Alternatively the immunogenicity of the graft may be reduced by preparing precursor cells from a donor with reduced antigenicity, such as transgenic animals which have altered or deleted MHC antigens.

Allografts

Grafting of precursor cells prepared from tissue which is allogeneic to that of the recipient will most often employ tissue typing in an effort to most closely match the histocompatibility typ of the recipient. Donor cell age as well as age of the recipient have been

demonstrated to be important factors in improving the probability of neuronal graft survival. The efficiency of grafting is reduced with increased age of donor cells. Furthermore, grafts are more readily accepted by younger recipients compared to older recipients. These two factors are likely to be as important for glial graft survival as they are for neuronal graft survival.

Implantation in Humans

Areas of demyelination in humans is generally associated with plaque like structures. Plaques can be visualized by magnetic resonance imaging. Accessible plaques are the target area for injection of NSCs. Standard stereotactic neurosurgical methods are used to inject cell suspensions both into the brain and spinal cord.

Remyelination by the injection of precursor cells is a useful therapeutic in a wide range of demyelinating conditions. It should also be borne in mind that in some circumstances remyelination by precursor cells will not result in permanent remyelination, and repeated injections will be required. Such therapeutic approaches offer advantage over leaving the condition untreated and may spare the recipient's life.

A list of human demyelinating diseases for which the cells of the present invention may provide treatment is as follows: disseminated perivenous encephalomyelitis, multiple sclerosis (Charcot and Marburg types), neuromyelitis optica, concentric sclerosis, acute, disseminated encephalomyelitides, post encephalomyelitis, postvaccinal encephalomyelitis, acute hemorrhagic leukoencephalopathy, progressive multifocal

leukoencephal pathy, idiopathic polyn uritis, diphtheric neuropathy, Pelizaeus-Merzbacher disease, neuromyelitis optica, diffuse cerebral sclerosis, central pontine myelinosis, spongiform leukodystrophy leukodystrophy (Alexander type).

Examples

Example 1
Propagation of precursor cells for transplantation

Embryonic day 15 (E15) Sprague Dawley rats are decapitated and the brain and striata are removed using sterile procedure. Tissue is mechanically dissociated with a fire-polished Pasteur pipette into serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (Gibco). The cells are centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in DMEM/F-12 medium for counting.

The cells are suspended in a serum-free medium composed of DMEM/F-12 (1:1) including glucose (0.6%), glutamine (2 μM), sodium bicarbonate (3 mM), and HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (5 mM) (all from Sigma except glutamine [Gibco]). A defined hormone mix and salt mixture (Sigma) that includes insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) is used in place of serum. In addition, the medium contains 16-20 ng/ml EGF (purified from mouse submaxillary, Collaborative Research) or TGFα (human recombinant, Gibco). The cells are seeded in a T25 culture flask and housed in an incubator at 37°C, 100% humidity, 95% air/5% CO_2. Cells proliferate within 3-4 days and, due to lack of substrate, lift off the floor of the flask and continue to proliferate in

suspension forming clusters of undifferentiated precursor cells known as neurospheres.

After 6-8 days in vitro (DIV) the neurospheres are removed, centrifuged at 400 r.p.m. for 2-5 minutes, and the pellet mechanically dissociated into individual cells with a fire-polished glass pasteur pipet. Cells are replated in the growth medium where proliferation of the stem cells and formation of new neurospheres is reinitiated. This procedure is repeated weekly and results in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of precursor cells is obtained.

Example 2
Remyelination of myelin deficient rats

Litters of first day postnatal myelin deficient rats are anesthetized using ice to produce hypothermia. Myelin deficiency is an X-linked trait and thus only one half of the males in any litter are affected. Therefore, only the males are used for these studies. Once anesthetized, a small rostral to caudal incision is made at the level of the lumbar enlargement. The muscle and connective tissue is removed to expose the vertebral laminae. Using a fine rat tooth forceps, one lamina at the lumbar enlargement is removed and a small cut is made in the dura mater to expose the spinal cord.

A stereotaxic device holding a glass pipet is used to inject a 1 μl aliquot of the cell suspension (approximately 50,000 cells/μl) described above. The suspension is slowly injected into a single site (although more could be done) in the dorsal columns of the spinal cord. As controls, some of the animals are sham-injected with sterile saline. The animals are

marked by clipping either toes or ears to distinguish between both experimental groups. Following injection of the cell suspension, the wound is closed using sutures or stainless steel wound clips and the animals are revived by warming on a surgical heating pad and then returned to their mother.

The animals are allowed to survive for three weeks post-injection and are then deeply anesthetized with nembutal (150 mg/kg) and perfused through the left ventricle. The tissues are then dissected from the animal and fixed for 1-3 days with 4% paraformaldehyde in PBS and 95% ethanol/5% acetic acid, respectively and then processed for epoxy embedding. One micron plastic sections are cut with an ultramicrotome and heat-sealed on glass microscope slides and either stained with alkaline toluidine blue (a histological stain for myelin) or processed for immunocytochemistry for the major myelin proteins. Because the myelin deficient rat spinal cord is almost completely devoid of myelin, myelin formed at or near the site of injection will be derived from the implanted cells. It is possible that the process of injection will allow for the entry of Schwann cells (myelinating cells of the peripheral nervous system) into the spinal cord. These cells are capable of forming myelin within the central nervous system but can be easily distinguished from oligodendrocytes using either light microscopy or immunocytochemistry for CNS myelin elements. As noted above, there is usually a very small amount of CNS myelin within the myelin deficient rat spinal cord. This myelin can be distinguished from normal donor myelin based on the mutation within the gene for the major CNS myelin protein, proteolipid protein (PLP).

The myelin deficient rat myelin is not immunoreactive for PLP while the donor myelin is.

The myelinated axons are found not only at the site of injection but also in adjacent vertebral sections indicating that the injected precursor cells both migrate away from the site of injection and differentiate to oligodendrocytes in order to form myelin.

Example 3

Remyelination in human Neuromyelitis optica

Neuromyelitis optica is a condition involving demyelination of principally the spinal cord and optic nerve. Onset is usually acute and in 50% of the cases death occurs within months. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Precursor cells are prepared from fetal human tissue by the method of Example 1. Cells are stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

Example 4

Remyelination in human Pelizaeus-Merzbacher disease

Pelizaeus-Merzbacher disease is a condition involving demyelination of the CNS. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Precursor cells are prepared from fetal human tissue by the method of Example 1. Cells are

stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

WHAT IS CLAIMED IS:

1. A method of remyelinating neurons comprising the steps of:

(a) isolating neural stem cells from the tissue of a donor,

(b) proliferating the isolated neural stem cells in a culture medium containing a growth factor to produce precursor cells,

(c) harvesting the precursor cells, and

(d) associating the harvested precursor cells with a demyelinated axon to effect remyelination.

2. The method of Claim 1 wherein the growth factor is epidermal growth factor.

3. The method of Claim 1 wherein the demyelinated axons are those of a recipient.

4. The method of Claim 1 wherein the precursor cells of step (b) are in neurospheres.

5. The method of Claim 3 wherein the donor is the recipient.

6. The method of Claim 3 wherein the recipient is human.

7. A method of remyelinating neurons comprising the steps of:

(a) isolating neural stem cells from the tissue of a donor,

(b) proliferating the isolated neural stem cells in a first culture medium containing a growth factor to produce precursor cells,

(c) differentiating the precursor cells in a second culture medium that is substantially free of said growth factor to produce oligodendrocytes, and

(d) associating the oligodendrocytes with a demyelinated axon to effect remyelination.

8. The method of Claim 7 wherein the culture medium of step (c) contains serum.

9. The method of Claim 7 wherein the growth factor is epidermal growth factor.

10. The method of Claim 7 wherein the association of step (d) occurs in the presence of type I astrocytes.

11. The method of Claim 7 wherein the association of step (d) occurs in the presence of platelet-derived growth factor.

12. The method of Claim 7 further comprising step of:

(e) adding type I astrocytes to the oligodendrocytes associated with the demyelinated axon.

13. The method of Claim 7 further comprising after step (d) the step of:

(e) adding platelet-derived growth factor to the oligodendrocytes associated with the demyelinated axon.

14. The method of Claim 7 wherein the precursor cells of step (b) are in neurospheres.

15. The method of Claim 7 wherein the demyelinated axons are those of a recipient.

16. The method of Claim 15 wherein the donor is the recipient.

17. The method of Claim 15 wherein the recipient is human.

18. A method for treating demyelinating disease comprising the steps of:

(a) isolating neural stem cells from the tissue of a donor,

(b) proliferating the isolated neural stem cells in a culture medium containing a growth factor to produce precursor cells,

(c) harvesting the precursor cells, and

(d) transplanting the harvested precursor cells to a position proximate to a demyelinated axon in a recipient having demyelinating disease to effect remyelination.

19. The method of Claim 18 wherein the growth factor is epidermal growth factor.

20. The method of Claim 18 wherein the disease is selected from the group consisting of multiple sclerosis, disseminated perivenous encephalomyelitis, neuromyelitis optica, concentric sclerosis, acute disseminated encephalomyelitides, post encephalomyelitis, acute hemorrhagic leukoencephalopathy, progressive multifocal leukoencephalopathy, idiopathic polyneuritis, diphtheric neuropathy, Pelizaeus-Merzbacher disease, neuromyelitis optica, diffuse cerebral sclerosis, central pontine myelinosis, and leukodystrophy.

21. A method for treating demyelinating dis ase comprising the steps of:

(a) isolating neural stem cells from the tissue of a donor,

(b) proliferating the isolated neural stem cells in a first culture medium containing a growth factor to produce precursor cells,

(c) differentiating the precursor cells in a second culture medium that is substantially free of said growth factor to produce oligodendrocytes, and

(d) transplanting the oligodendrocytes into a recipient having demyelinating disease.

22. The method of claim 21 wherein the culture medium of step (c) contains serum.

23. The method of Claim 21 wherein the growth factor is epidermal growth factor.

24. The method of Claim 21 wherein the disease is selected from the group consisting of multiple sclerosis, disseminated perivenous encephalomyelitis, neuromyelitis optica, concentric sclerosis, acute disseminated encephalomyelitides, post encephalomyelitis, acute hemorrhagic leukoencephalopathy, progressive multifocal leukoencephalopathy, idiopathic polyneuritis, diphtheric neuropathy, Pelizaeus-Merzbacher disease, neuromyelitis optica, diffuse cerebral sclerosis, central pontine myelinosis, and leukodystrophy.

25. A method of producing glial cells comprising the steps of:

(a) isolating neural stem cells from a donor,

(b) proliferating the isolated neural stem cells in a first culture medium containing a growth factor to produce precursor cells, and

(c) differentiating the precursor cells in a second culture medium that is substantially free of said growth factor to obtain glial cells.

26. The method of Claim 25 wherein the culture medium of step (c) contains serum.

27. The method of Claim 25 wherein the glial cells are oligodendrocytes.

28. The method of claim 25 wherein the glial cells are astrocytes.

29. The method of Claim 25 wherein the growth factor is epidermal growth factor.

30. The method of Claim 25 wherein the precursor cells of step (b) are in neurospheres.

31. Glial cells formed by the method of Claim 25.

32. A precursor cell <u>in vitro</u> living in a culture medium having a growth factor.

33. An oligodendrocyte derived from a precursor cell and living in a culture medium.

34. An astrocyte derived from a precursor cell and living in a culture medium.

35. A remyelinated neuron formed by the method of claim 1.

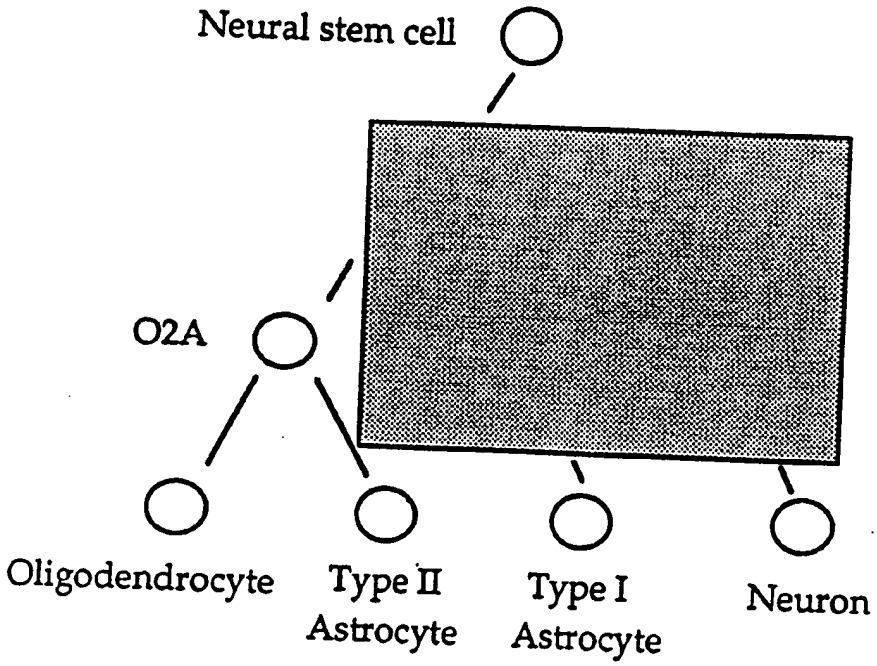
Neural stem cell
O2A
Oligodendrocyte
Type II Astrocyte
Type I Astrocyte
Neuron

Figure 1

INTERNATIONAL SEARCH REPORT

Inten ıal Application No
PCT/CA 93/00428

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N5/08 C12N5/00 A61K35/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE vol. 255 , 27 March 1992 , WASHINGTON D.C.,USA pages 1707 - 1710 REYNOLDS ET AL 'GENERATION OF NEURONS AND ASTROCYTES FROM ISOLATED CELLS OF THE ADULT MAMMALIAN CENTRAL NERVOUS SYSTEM' cited in the application	25-34
Y	see page 1707,summary see page 1709, paragraph 3 -paragraph 6 --- -/--	1-24,35

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1993	24 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 93/00428

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FILE SERVER STN KARLSRUHE,FILE BIOSIS ABSTRACT NO.91:324328 & RADIOTHER ONCOL 20 (4) 1991 258-264	25-34
Y	VAN DER MAAZEN ET AL:'RADIOSENSITIVITY OF GLIAL PROGENITOR CELLS OF THE PERINATAL AND ADULT RAT OPTIC NERVE STUDIED BY AN IN-VITRO CLONOGENIC ASSAY see abstract	1-24,35
X	WO,A,91 02003 (BOARD OF REGENTS,THE UNIVERSITY OF TEXAS SYSTEM) 21 February 1991 see page 18, line 11 - page 28, line 36	32-34
X	WO,A,91 09936 (HANA BIOLOGICS,INC.) 11 July 1991	32
A	see the whole document	
Y	THE JOURNAL OF CELL BIOLOGY vol. 109, no. 5 , November 1989 , NEW YORK,USA pages 2405 - 2416 GODFRAIND ET AL 'IN VIVO ANALYSIS OF GLIAL CELL PHENOTYPES DURING A VIRAL DEMYELINATING DISEASE IN MICE' cited in the application see page 2405,abstract see page 2406, paragraph 3 see page 2413, paragraph 3 - page 2414, paragraph 2 see page 2415, paragraph 3	1-24,35
P,X	WO,A,93 01275 (WEISS ET AL) 21 January 1993 see the whole document	25-34

1

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/CA93/00428

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 18-24 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter ...al Application No
PCT/CA 93/00428

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9102003	21-02-91	AU-A- 6174490	11-03-91
WO-A-9109936	11-07-91	NONE	
WO-A-9301275	21-01-93	AU-A- 2242592	11-02-93